Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Microbiology

Metabolomic analysis of *Entamoeba*: applications and implications

Ghulam Jeelani[1] and Tomoyoshi Nozaki[1,2]

Entamoeba histolytica is an enteric protozoan parasite that causes hemorrhagic dysentery and extraintestinal abscesses in millions of inhabitants of endemic areas. The genome of *E. histolytica* has already been sequenced and used to predict the metabolic potential of the organism. Since nearly 56% of the *E. histolytica* genes remain unannotated, correlative 'omics' analyses of genomics, transcriptomics, proteomics, and biochemical metabolic profiling are essential in uncovering new, or poorly understood metabolic pathways. Metabolomics aims at understanding biology by comprehensive metabolite profiling. In this review, we discuss recent metabolomics approaches to elucidate unidentified metabolic systems of this pathogen and also discuss future applications of metabolomics to understand the biology and pathogenesis of *E. histolytica.*

Addresses
[1] Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan
[2] Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

Corresponding author: Nozaki, Tomoyoshi (nozaki@nih.go.jp)

Current Opinion in Microbiology 2014, **20**:118–124

This review comes from a themed issue on **Host-microbe interactions: parasites**

Edited by **Manoj Duraisingh** and **Nancy Guillén**

For a complete overview see the Issue and the Editorial

Available online 18th June 2014

http://dx.doi.org/10.1016/j.mib.2014.05.016

Introduction

Amebiasis is a disease caused by a unicellular parasitic protozoan *E. histolytica*. The World Health Organization estimates that approximately 50 million people worldwide suffer from invasive amebic infection, resulting in 40 to 100 thousand deaths annually [1]. Only metronidazole and related compounds are commonly used against invasive intestinal and extraintestinal amebiasis [2]. Although clinical resistance against metronidazole has not yet been demonstrated, a few limitations including low efficacy against asymptomatic cyst carriers and sporadic cases of treatment failure have been reported [2]. In addition, it has been shown that this parasite adapts to subtherapeutic levels of metronidazole in vitro [3]. Therefore, the development of a novel prophylactics and chemotherapeutics to control amebic infections is required and to this end, identification of novel essential pathways and enzymes that are expressed only under specific physiological conditions, is particularly needed.

The draft genome of *E. histolytica* was first published in 2005 [4]. New assembly and reannotation of the *E. histolytica* genome was published in 2010 [5]. Recently, Ehrenkaufer *et al.* reported the sequencing and assembly of the genome of a reptilian *E. invadens* species, which produces symptoms similar to *E. histolytica* in humans [6••]. Based upon genome data, *E. histolytica* metabolic pathways were predicted and tentatively reconstructed [7]. However, it is not easy to comprehensively understand a full metabolic capacity of the parasite. This is mainly because (1) annotation of genes and pathways are not always faithful and novel proteins and pathways are occasionally ignored, (2) genes in the genome are not always simultaneously expressed, but often expressed in developmental stage-dependent or condition (e.g. stresses and nutrients)-dependent fashions, and (3) metabolisms are regulated at transcriptional, post-transcriptional, post-translational, and cellular levels (e.g. antisense small RNA, phosphorylation, feedback, and compartmentalization). To examine a cell's complete metabolism, a new discipline, termed metabolomics, which aims to catalog all the metabolites in a given cell, tissue, organ, or organism at any given time, has been developed [8].

Metabolomic analysis has been widely applied to study the systems biology of numerous model organisms, including archeae [9], eubacteria [10], fungi [11], plants [12], animals [13], and human cell tissue cultures [14]. For protozoan parasites, metabolomics has been used to study host–parasite interaction of trypanosomatid parasites (see review by Creek *et al.* [15••]). Metabolomics studies have been carried out in *Leishmania* (see [16] for review), *Trypanosoma* (for review see [17]), and *Plasmodium* (see review by Laksmanan *et al.* [18]). For instance, metabolomic approaches have been used to identify stage-specific and species-specific differences in the metabolic networks of *Leishmania* [19]. Using ^{13}C-labeled carbon sources and gas chromatography–mass spectrometry (GC–MS) approach, Saunders *et al.* revealed that *L. mexicana* promastigotes are dependent on succinate fermentation to balance the energy and redox state of glycosomes, and intriguingly, to maintain mitochondrial TCA cycle anaplerosis [19]. Recently Cobbold *et al.* [20] applied a rapid stable-isotopic labeling technique in combination with high resolution mass

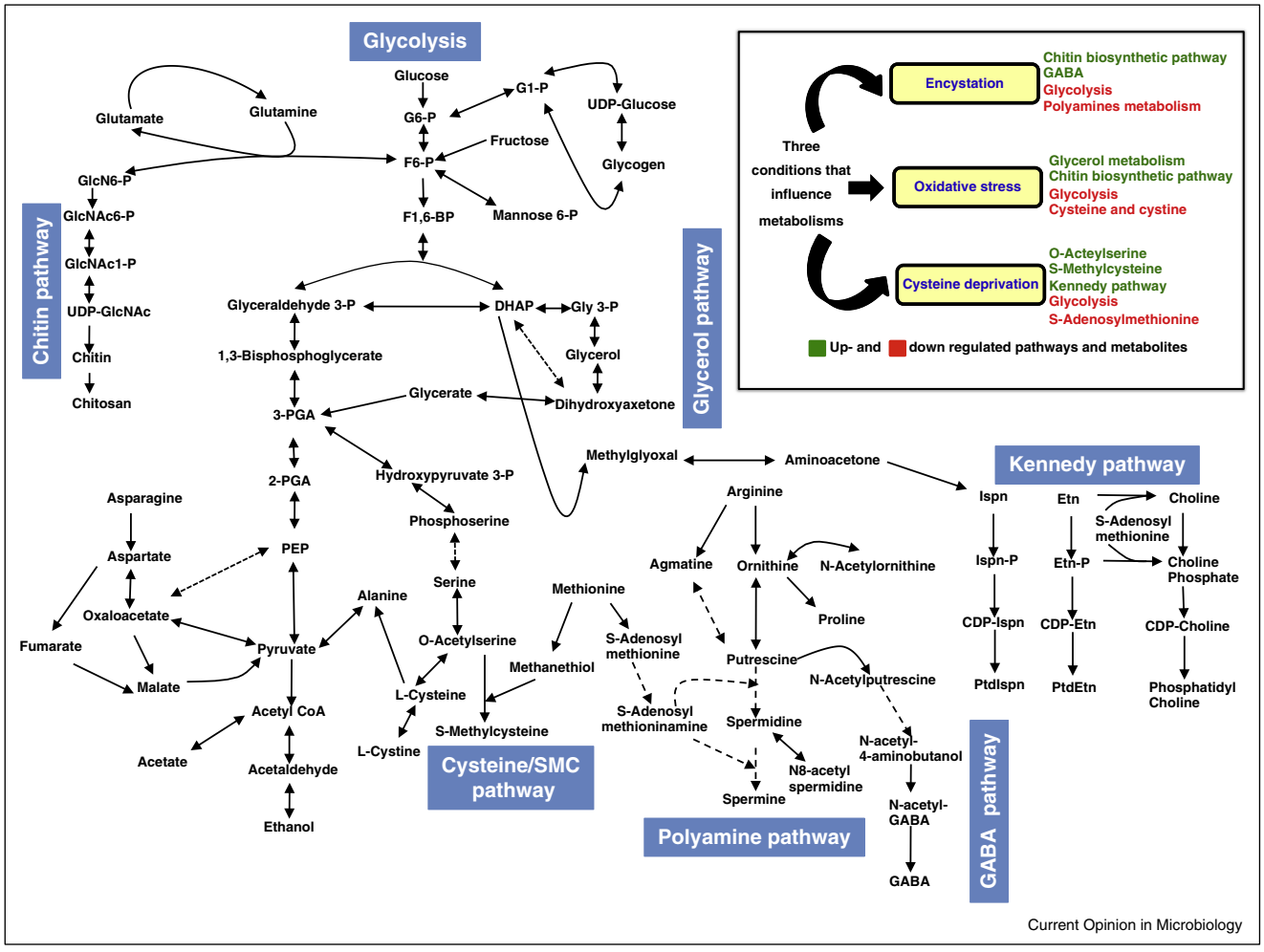
spectrometry to study blood-stage acetyl-CoA metabolism of the human malaria parasites *P. falciparum*. They found that the pyruvate dehydrogenase-like enzyme, likely the branched-chain keto acid dehydrogenase complex, contributes glucose-derived acetyl-CoA to the TCA cycle in a stage-independent process, whereas anaplerotic carbon enters the TCA cycle via a stage-dependent phosphoenolpyruvate carboxylase/phosphoenolpyruvate carboxykinase process. This study highlights the parasite's ability to restructure metabolism to meet its developmental requirements. For metabolomics analysis of parasitic protozoa in general, we recommend to read a very comprehensive review published by Paget *et al.* [21••].

In this review we will focus on our recent discoveries of global changes in *Entamoeba* metabolism in response to environmental stresses and during stage transition by targeted metabolome analysis. Key findings of these studies are summarized in Figure 1. Application of the metabolomics-based approaches discussed in this review marks the first step toward systems biology-based understanding of *Entamoeba* metabolism and biology.

Central energy and amino acid metabolism in *Entamoeba*: background

E. histolytica trophozoites are microaerophilic, and shown to consume oxygen and tolerate low levels of oxygen pressure [22]. *E. histolytica* lacks the general form of

Figure 1



Predicted metabolic networks of *Entamoeba* based on metabolomic analyses in responses to three representative conditions, that is encystation, oxidative stress, and cysteine deprivation. Solid lines represent the steps catalyzed by the enzymes whose encoding genes are present in the genomes, whereas dashed lines indicate those likely absent in the genome or not identified so far. Abbreviations are: G6-P, glucose 6-phosphate; G1-P, glucose 1-phosphate; F6-P, fructose 6-phosphate; F1,6-BP, fructose 1,6-biphosphate; DHAP, dihydroxy acetone phosphate; Gly 3-P, glycerol 3-phosphate; PGA, phosphoglycerate; PEP, phosphoenolpyruvate; GlcN6P, glucosamine 6-phosphate; GlcNAc6-P, N-acetylglucosamine 6-phosphate; GlcNAc1-P, N-acetyl glucosamine 1-phosphate; UDP-GlcN6P, UDP-glucosamine 6-phosphate; GABA, γ-aminobutyric acid; SMC, S-methylcysteine; Ispn, isopropanolamine; Ispn-P, isopropanolamine phosphate; PtdIspn, phosphatidylisopropanolamine; Etn, ethanolamine; Etn-P, ethanolamine phosphate; and PtdEtn, phosphatidylethanolamine.

mitochondria as found in the aerobic eukaryotes, but instead possesses a highly divergent mitochondrion-related organelle, named mitosomes [23]. *E. histolytica* mitosomes lack features of aerobic energy metabolism including the tricarboxylic acid (TCA) cycle and oxidative phosphorylation, and energy generation is primarily dependent on substrate level phosphorylation in glycolysis and fermentation, which occur in the cytosol [24]. In *E. histolytica*, pyruvate is converted to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (PFOR), and acetyl-CoA is either converted to acetate with a concomitant ATP generation or reduced to ethanol with regeneration of NAD [25]. Glycolysis is the only pathway in *E. histolytica* for which kinetic parameters of enzymes have been comprehensively investigated [26–28]. Recently Pineda *et al.* reported that under aerobic conditions the bifunctional aldehyde–alcohol dehydrogenase exerts significant flux control on ethanol and acetate production in *E. histolytica* [29•].

Earlier studies using ^{14}C-labeled glucose showed that more than 95% of labeled glucose was converted to glycogen, carbon dioxide, ethanol, and acetate [30]. Only a trace amount of label was found in protein, lipid, and nucleic acid. Bakker-Grunwald *et al.* using ^{13}C NMR spectroscopy identified various abundant metabolites in *E. histolytica* [31]. Besides glycolysis, the catabolism of amino acids also contributes to the generation of ATP. It was demonstrated that in the absence of glucose, *E. histolytica* and *E. invadens* preferentially consume several amino acids (asparagine, arginine, leucine, threonine, and serine) [32]. Baumel-Alterzon *et al.* demonstrated using DNA microarray that *E. histolytica* trophozoites that were adapted to grow in the absence of glucose showed remarkable changes in the gene expression of dihydropyrimidine dehydrogenase, concomitant with increase in the virulence [33••]. Sulfur-containing amino acid metabolism is also unique in this parasite. Despite the fact that amino acid metabolism is largely reduced in the majority of parasitic protozoa, unique sulfur-containing amino acid metabolism are highly operational in *E. histolytica* such as de novo L-cysteine/S-methylcysteine (SMC) biosynthesis and methione/homocysteine/cysteine degradation by methionine γ-lyase (MGL) [34].

Application of metabolomic analysis to the understanding of encystation (stage conversion)

Stage conversion is often accompanied by most drastic biochemical and morphological changes. The transition from the motile proliferating disease-causing trophozoites to the dormant cyst form, named encystation, is essential for parasite survival in the environment and transmission. Jeelani *et al.* recently investigated metabolic and transcriptomic changes that occur during encystation in *E. invadens*, which is a relative of *E. histolytica* from reptiles, and, unlike *E. histolytica*, encysts in *in vitro* culture, using capillary electrophoresis/time-of-flight mass spectrometry (CE-ToFMS) and DNA microarray analysis [35••]. It was demonstrated that as encystation progresses, the levels of a majority of metabolites involved in glycolysis as well as all nucleotides decreased markedly. Furthermore, intermediates for chitin biosynthesis dramatically increased (Figure 1). These findings agree very well with the notion created by numerous previous works: energy generation and storage ceases during and after encystation, and the flux of glucose utilization shifts from glycolysis and fermentation to chitin wall biosynthesis.

Surprisingly, intermediates of polyamine metabolism showed remarkable changes during encystation. These intermediates include putrescine, spermidine, spermine, and N^8-acetylspermidine, all of which were present in trophozoites. Furthermore, the levels of these metabolites dramatically decreased and they were almost deprived as encystation proceeded. Although there is a possibility that polyamines are incorporated from the culture medium, these findings led us to presume that *Entamoeba* is capable of metabolizing these polyamines during encystation, and the polyamine biosynthetic or scavenging pathways exist in this organism. The fate of polyamines is not well understood, but some polyamines appear to be secreted by trophozoites (unpublished). The presence of polyamine metabolism was totally unexpected because several genes encoding key enzymes such as S-adenosylmethionine decarboxylase, spermidine synthase, and spermine synthase, known to be involved in polyamine biosynthesis in other organisms, have not been detected in the *Entamoeba* genome [4].

Second unexpected discovery was the production of γ-aminobutyric acid (GABA). In other organisms like bacteria, fungi, mammals, and plants GABA is made from L-glutamate in a single reaction catalyzed by glutamate decarboxylase [36], which is missing in the *Entamoeba* genome [4]. Thus, amino acid decarboxylases encoded in the genome may play the equivalent role. Further investigations of the time kinetics of the changes in various metabolites indicate that GABA is synthesized by removal of the acetyl moiety from N-acetylputrescine (Figure 1). GABA is a major inhibitory neurotransmitter in the mammalian central nervous system. Role of GABA during encystation is not clear. However, in *Dictyostelium discoideum*, a soil-living ameba, GABA has been shown to induce terminal differentiation (sporulation) through $GABA_B$ receptor [37]. Further investigations such as fluxomics using isotopic labeling should unveil the exact pathway and enzymes involved in the formation of GABA.

Another important and totally unexpected finding revealed by metabolomics was the production of biogenic amines, namely cadaverine, isoamylamine, and isobutylamine, during encystation. Interestingly, the increase of

individual biogenic amines was transient; biogenic amines were increased only in the early phase of encystation (before apparent morphological and biochemical changes), when the trophozoites formed large multicellular aggregates (precyst), suggesting their role as signaling molecules. Enzymes involved in the decarboxylation of corresponding amino acids have not been identified, while a gene encoding putative arginine/lysine/ornithine decarboxylase is present. Future work is needed to delineate whole genetic and metabolic networks that regulate synthesis and degradation of these metabolites and their precise role in encystation.

Application of metabolomic analysis to the understanding of oxidative stresses

Experimental conditions such as various nutrient sources and stresses have been commonly used in well-characterized microorganisms like *E. coli* [38]. Similarly, *E. histolytica* trophozoites were also tested for such environmental conditions to understand the response [39]. *E. histolytica* trophozoites are exposed to various reactive oxygen or nitrogen species (ROS and RNS) during tissue invasion, colonization in the intestine, and extra intestinal propagation [39]. *E. histolytica* lacks most of the components involved in the usual antioxidant defense mechanisms in aerobic organisms, such as catalase, glutathione, and the glutathione-recycling enzymes. *E. histolytica* also lacks glucose 6-phosphate dehydrogenase (G6PD), and thus functional pentose phosphate pathway is absent to yield NADPH [4]. However, the genome of *E. histolytica* encodes several proteins for detoxification of reactive oxygen and nitrogen species (ROS, RNS), such as peroxiredoxin, superoxide dismutase, rubrerythrin, hybrid-cluster protein, and flavo-di-iron proteins [40,41]. In addition, *E. histolytica* also possesses pyridine nucleotide transhydrogenase, and NADH kinase for NADPH synthesis from NAD(H) [42,43•]. NAPDH is in turn utilized in the detoxification of ROS and RNS. Recently Pearson *et al.* utilized DNA affinity chromatography and mass spectrometry to identify a new *E. histolytica* transcription factor that plays a role in coordinately regulating gene expression in response to hydrogen peroxide exposure [44••].

Response to H_2O_2-mediated and paraquat-mediated oxidative stress

Husain *et al.* [45••] investigated global metabolic responses in response to H_2O_2-mediated and paraquat-mediated oxidative stress in *E. histolytica* trophozoites. In this study, they showed that oxidative stress caused changes in the metabolites involved in glycolysis and its associated pathways, as evidenced by the accumulation of glycolytic intermediates upstream of pyruvate, and reduced ethanol production. They have further shown that oxidative stress inactivated several key enzymes of glycolysis and its associated pathways such as PFOR, phosphoglycerate mutase, and NAD^+-dependent alcohol dehydrogenase, and resulted in the inhibition of glycolysis and the redirection of metabolic flux toward glycerol production, chitin biosynthesis, and the non-oxidative branch of the pentose phosphate pathway. As a result of the repression of glycolysis and fermentation, the levels of nucleoside triphosphates were decreased upon H_2O_2 stress, whereas the levels of nucleoside monophosphates increased, in a manner opposite to the decrement in their corresponding triphosphate counterparts. Both paraquat and H_2O_2-mediated oxidative stress led to a decrement in L-cysteine and L-cystine, and a slight increment in cysteine S-sulfinate, in a time-dependent manner. These findings suggest that L-cysteine is involved in scavenging of ROS in *E. histolytica*.

Metabolomic analysis in this study further demonstrated the presence of functional glycerol biosynthetic pathway (Figure 1) and functional glycerol 3-phosphate (Gly 3-P) dehydrogenase (G3PDH) in this parasite. Gly 3-P was one of the most highly affected metabolites upon oxidative stress. It was speculated that dihydroxyacetone phosphate, but not Gly 3-P, is mainly used for triglyceride synthesis, because Gly 3-P yields ATP by glycerate kinase. This could be the primary reason of the redirection of the glycolytic pathway upon oxidative stress. However, the physiological significance of the increased production of Gly 3-P and glycerol upon oxidative stress need to be demonstrated.

L-cysteine deprivation

L-cysteine is the major low molecular weight thiol in *E. histolytica* trophozoites and has been implicated in the survival, growth, attachment, elongation, motility, gene regulation, and antioxidative stress defense of this organism [46–49]. *In vitro* growth of amebic trophozoites requires high concentrations of L-cysteine, which can be replaced by D-cysteine, L-cystine, or L-ascorbic acid, indicating that the extracellular cysteine/cystine or thiols have an indispensable role in parasite growth [50].

Under L-cysteine deprivation, two metabolites that had never been demonstrated in *E. histolytica*, S-methyl cysteine (SMC) and O-acetylserine (OAS), were shown to be accumulated [51]. SMC is a sulfur-containing amino acid that was never detected in protozoa, but is widely present in relatively large amounts in several legumes, where it is considered to serve as sulfur storage [52]. OAS was presumed to be present in *E. histolytica* as it is a substrate for the de novo synthesis of L-cysteine/SMC (Figure 1). However, it was never demonstrated because its steady state concentrations are kept very low due to the product inhibition of serine acetyltransferase by L-cysteine, or OAS has a short half-life, due to immediate conversion to N-acetylserine. This study unequivocally demonstrated that the pathway previously considered to serve to produce L-cysteine de novo, is mainly involved in

the production of SMC, the fate and physiological significance of which are not yet fully understood.

L-cysteine depletion also resulted in reduced levels of S-adenosylmethionine (SAM). SAM is, in general, a precursor for polyamine biosynthesis and the essential methyl donor for numerous transmethylation reactions, including DNA methylation [53]. L-cysteine also regulates the Kennedy pathway (Figure 1), the major pathway for phospholipid biosynthesis. L-cysteine deprivation resulted in the accumulation of an unusual phospholipid, phosphatidylisopropanolamine, and also affected the composition and ratio of the major phospholipids [51]. Under L-cysteine-depleted conditions, the synthesis of isopropanolamine, isopropanolamine phosphate, ethanolamine phosphate, and choline phosphate was elevated, while phosphatidylethanolamine synthesis was inhibited. These findings indicate dramatic modulations in phospholipid metabolism under L-cysteine deprivation. Further investigation is needed to understand the physiological role of phosphatidylisopropanolamine, its derivatives, and related pathways, which are potentially a new attractive drug target for the development of new chemotherapeutics against amebiasis.

Conclusion and perspective

Elaborate investigation at the metabolic level is particularly important to understand role of metabolism in parasitism and pathogenesis, and also to develop new chemotherapies against pathogens, in which metabolic processes dissimilar to those from humans are reasonable drug targets. Furthermore, as profound metabolic disturbances often underlie the microbicidal effects of drugs, the mode of drug action can be easily and rationally probed by metabolomics approaches. Metabolomics also allows elucidation of the mechanisms of drug resistance of parasites. Discoveries of new metabolic biomarkers in infected humans may also yield reliable diagnostic measures (diagnostics), predictors of treatment outcome (e.g. drug resistance), and related clinical polymorphisms (e.g. severity of disease, tissue tropisms). An understanding of metabolic networks of parasites will be further improved by combining metabolomic analysis with other profiling technologies, especially proteomics, and integrative techniques like metabolic control analysis [54].

Future applications of metabolomics toward an understanding of *Entamoeba* metabolism also include metabolic profiling of the organelles. Metabolomic analysis of mitosomes is of particular interest because of their unique unprecedented compartmentalization of sulfate activation. Metabolomics should in general unveil metabolic roles of the highly divergent mitochondrion-related organelles in anaerobic eukaryotes (see review by Makiuchi *et al.*) [55••]. In summary, metabolomics-based approaches are practically the best solution toward reclamation of new areas of *Entamoeba* metabolism and biochemistry.

Acknowledgements

Our works summarized in this review were supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) (23117001, 23117005, 23390099), a grant from Global COE Program from MEXT, a grant for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare of Japan, a grant for research to promote the development of anti-AIDS pharmaceuticals from the Japan Health Sciences Foundation (KHA1101) to T.N.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Ximénez C, Morán P, Rojas L, Valadez A, Go mez A: **Reassessment of the epidemiology of amebiasis: state of the art**. *Infect Gen Evol* 2009, **9**:1023-1032.

2. Ali V, Nozaki T: **Current therapeutics, their problems, and sulfur-containing amino acid metabolism as a novel target against infections by "amitochondriate" protozoan parasites**. *Clin Microbiol Rev* 2007, **20**:164-187.

3. Wassmann C, Hellberg A, Tannich E, Bruchhaus I: **Metronidazole resistance in the protozoan parasite *Entamoeba histolytica* is associated with increased expression of iron-containing superoxide dismutase and peroxiredoxin and decreased expression of ferredoxin 1 and flavin reductase**. *J Biol Chem* 1999, **274**:26051-26056.

4. Loftus B, Anderson I, Davies R, Alsmark UC, Samuelson J, Amedeo P, Roncaglia P, Berriman M, Hirt RP, Mann BJ *et al.*: **The genome of the protist parasite *Entamoeba histolytica***. *Nature* 2005, **433**:865-868.

5. Lorenzi HA, Puiu D, Miller JR, Brinkac LM, Amedeo P, Hall N, Caler EV: **New assembly, reannotation and analysis of the *Entamoeba histolytica* genome reveal new genomic features and protein content information**. *PLoS Negl Trop Dis* 2010, **4**:e716.

6. •• Ehrenkaufer GM, Weedall GD, Williams D, Lorenzi HA, Caler E, Hall N, Singh U: **The genome and transcriptome of the enteric parasite *Entamoeba invadens*, a model for encystation**. *Genome Biol* 2013, **14**:R77.
In this publication the authors carried out genome and transcriptome analyses of *E. invadens* during stage conversion and demonstrate that a number of core processes are common to encystation between distantly related parasites.

7. Clark CG, Alsmark UC, Tazreiter M, Saito-Nakano Y, Ali V, Marion S, Weber C, Mukherjee C, Bruchhaus I *et al.*: **Structure and content of the *Entamoeba histolytica* genome**. *Adv Parasitol* 2007, **65**:51-190.

8. Dixon RA, Strack D: **Phytochemistry meets genome analysis, and beyond**. *Phytochemistry* 2003, **62**:815-816.

9. Trauger SA, Kalisak E, Kalisiak J, Morita H, Weinberg MV, Menon AL, Poole FL II, Adams MW, Siuzdak G: **Correlating the transcriptome, proteome, and metabolome in the environmental adaptation of a hyperthermophile**. *J Proteome Res* 2008, **7**:1027-1035.

10. Rabinowitz JD: **Cellular metabolomics of *Escherichia coli***. *Expert Rev Proteomics* 2007, **4**:187-198.

11. Brauer MJ, Yuan J, Bennett BD, Lu W, Kimball E, Botstein D, Rabinowitz JD: **Conservation of the metabolomic response to starvation across two divergent microbes**. *Proc Natl Acad Sci USA* 2006, **103**:19302-19307.

12. Cho K, Shibato J, Agrawal GK, Jung YH, Kubo A, Jwa NS, Tamogami S, Satoh K, Kikuchi S, Higashi T *et al.*: **Integrated transcriptomics, proteomics, and metabolomics analyses to**

survey ozone responses in the leaves of rice seedling. *J Proteome Res* 2008, **7**:2980-2998.

13. Sun G, Yang K, Zhao Z, Guan S, Han X, Gross RW: **Shotgun metabolomics approach for the analysis of negatively charged water-soluble cellular metabolites from mouse heart tissue**. *Anal Chem* 2007, **79**:6629-6640.

14. Khoo SH, Al-Rubeai M: **Metabolomics as a complementary tool in cell culture**. *Biotechnol Appl Biochem* 2007, **47**:71-84.

15. •• Creek D, Anderson J, McConville M, Barrett M: **Metabolomic analysis of trypanosomatid protozoa**. *Mol Biochem Parasitol* 2012, **181**:73-84.
In this review, the authors highlighted metabolomics approaches applied to the kinetoplastid parasites (*Trypanosoma brucei* and *Leishmania* species).

16. Scheltema R, Decuypere S, T'Kindt R, Dujardin J, Coombs G, Breitling R: **The potential of metabolomics for *Leishmania* research in the post-genomics era**. *Parasitology* 2010, **137**:1291-1302.

17. Barrett MP, Bakker BM, Breitling R: **Metabolomic systems biology of trypanosomes**. *Parasitology* 2010, **137**:1285-1290.

18. Lakshmanan V, Rhee K, Daily J: **Metabolomics and malaria biology**. *Mol Biochem Parasitol* 2011, **175**:104-111.

19. Saunders EC, Ng WW, Chambers JM, Ng M, Naderer T, Krömer JO, Likic VA, McConville MJ: **Isotopomer profiling of *Leishmania mexicana* promastigotes reveals important roles for succinate fermentation and aspartate uptake in tricarboxylic acid cycle (TCA) anaplerosis, glutamate synthesis, and growth**. *J Biol Chem* 2011, **286**:27706-27717.

20. Cobbold SA, Vaughan AM, Lewis IA, Painter HJ, Camargo N, Perlman DH, Fishbaugher M, Healer J, Cowman AF, Kappe SH, Llinás M: **Kinetic flux profiling elucidates two independent acetyl-CoA biosynthetic pathways in *Plasmodium falciparum***. *J Biol Chem* 2013, **288**:36338-36350.

21. •• Paget T, Haroune N, Bagchi S, Jarroll E: **Metabolomics and protozoan parasites**. *Acta Parasitol* 2013, **58**:127-131.
An insightful review describing the state-of-the-art technologies in the well-established area of metabolomics in protozoan parasites.

22. Weinbach EC, Diamond LS: ***Entamoeba histolytica*. I. Aerobic metabolism**. *Exp Parasitol* 1974, **35**:232-243.

23. Tovar J, Fischer A, Clark CG: **The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica***. *Mol Microbiol* 1999, **32**:1013-1021.

24. Reeves RE: **Metabolism of *Entamoeba histolytica* Schaudinn, 1903**. *Adv Parasitol* 1984, **23**:105-142.

25. Reeves RE, Warren LG, Susskind B, Lo HS: **An energy conserving pyruvate-to-acetate pathway in *Entamoeba histolytica*. Pyruvate synthase and a new acetate thiokinase**. *J Biol Chem* 1977, **252**:726-731.

26. Saavedra E, Encalada R, Pineda E, Jasso-Chavez R, Moreno-Sanchez R: **Glycolysis in *Entamoeba histolytica*. Biochemical characterization of recombinant glycolytic enzymes and flux control analysis**. *FEBS J* 2005, **272**:1767-1783.

27. Saavedra E, Marín-Hernández A, Encalada R, Olivos A, Mendoza-Hernández G, Moreno-Sánchez R: **Kinetic modeling can describe in vivo glycolysis in *Entamoeba histolytica***. *FEBS J* 2007, **274**:4922-4940.

28. Moreno-Sánchez R, Encalada R, Marín-Hernández A, Saavedra E: **Experimental validation of metabolic pathway modeling. An illustration with glycolytic segments from *Entamoeba histolytica***. *FEBS J* 2008, **275**:3454-3469.

29. • Pineda E, Encalada R, Olivos-García A, Néquiz M, Moreno-Sánchez R, Saavedra E: **The bifunctional aldehyde–alcohol dehydrogenase controls ethanol and acetate production in *Entamoeba histolytica* under aerobic conditions**. *FEBS Lett* 2013, **587**:178-184.
This study showed that bifunctional aldehyde–alcohol dehydrogenase exerts significant flux-control on the carbon end-product formation in ameba trophozoites subjected to aerobic conditions.

30. Montalvo FE, Reeves RE, Warren LG: **Aerobic and anaerobic metabolism in *Entamoeba histolytica***. *Exp Parasitol* 1971, **30**:249-256.

31. Bakker-Grunwald T, Martin JB, Klein G: **Characterization of glycogen and amino acid pool of *Entamoeba histolytica* by ^{13}C-NMR spectroscopy**. *J Eukaryot Microbiol* 1995, **42**:346-349.

32. Zuo X, Coombs GH: **Amino acid consumption by the parasitic, amoeboid protists *Entamoeba histolytica* and *E. invadens***. *FEMS Microbiol Lett* 1995, **130**:253-258.

33. •• Baumel-Alterzon S, Weber C, Guillén N, Ankri S: **Identification of dihydropyrimidine dehydrogenase as a virulence factor essential for the survival of *Entamoeba histolytica* in glucose-poor environments**. *Cell Microbiol* 2013, **15**:130-144.
This paper described a new role of dihydropyrimidine dehydrogenase in promoting the survival of *E. histolytica* trophozoites in a glucose-poor environment using DNA microarray-based transcriptomic analysis.

34. Nozaki T, Ali V, Tokoro M: **Sulfur-containing amino acid metabolism in parasitic protozoa**. *Adv Parasitol* 2005, **60**:1-99.

35. •• Jeelani G, Sato D, Husain A, Escueta-de Cadiz A, Sugimoto M, Soga T, Suematsu M, Nozaki T: **Metabolic profiling of the protozoan parasite *Entamoeba invadens* revealed activation of unpredicted pathway during encystation**. *PLoS One* 2012, **7**:e37740.
This study unveiled the dynamics of the transcriptional and metabolic regulatory networks during encystation, using CE-TOFMS-based metabolite profiling and DNA microarray-based gene expression profiling.

36. Bown AW, Macgregor KB, Shelp BJ: **Gamma-aminobutyrate: defense against invertebrate pests?** *Trends Plant Sci* 2006, **11**:424-427.

37. Anjard C, Loomis WF: **GABA induces terminal differentiation of Dictyostelium through a GABAB receptor. 2006**. *Development* 2006, **133**:2253-2261.

38. Brauer MJ, Yuan J, Bennett BD, Lu W, Kimball E, Botstein D, Rabinowitz JD: **Conservation of the metabolomic response to starvation across two divergent microbes**. *Proc Natl Acad Sci U S A* 2006, **103**:19302-19307.

39. Vicente JB, Ehrenkaufer GM, Saraiva LM, Teixeira M, Singh U: ***Entamoeba histolytica* modulates a complex repertoire of novel genes in response to oxidative and nitrosative stresses: implications for amebic pathogenesis**. *Cell Microbiol* 2009, **11**:51-69.

40. Saraiva LM, Vicente JB, Teixeira M: **The role of the flavodiiron proteins in microbial nitric oxide detoxification**. *Adv Microb Physiol* 2004, **49**:77-129.

41. Sen A, Chatterjee NS, Akbar MA, Nandi N *et al.*: **The 29-kilodalton thiol-dependent peroxidase of *Entamoeba histolytica* is a factor involved in pathogenesis and survival of the parasite during oxidative stress**. *Eukaryot Cell* 2007, **6**:664-673.

42. Yousuf MA, Mi-ichi F, Nakada-Tsukui K, Nozaki T: **Localization and targeting of an unusual pyridine nucleotide transhydrogenase in *Entamoeba histolytica***. *Eukaryot Cell* 2010, **9**:926-933.

43. • Jeelani G, Husain A, Sato D, Soga T, Suematsu M, Nozaki T: **Biochemical and functional characterization of novel NADH kinase in the enteric protozoan parasite *Entamoeba histolytica***. *Biochimie* 2013, **95**:309-319.
In this study the authors demonstrated that NAD(H) kinase is involved in the maintenance of the cellular NADP(H) pool and also plays an important role in response to, and survival under oxidative stress.

44. •• Pearson RJ, Morf L, Singh U: **Regulation of H_2O_2 stress-responsive genes through a novel transcription factor in the protozoan pathogen *Entamoeba histolytica***. *J Biol Chem* 2013, **288**:4462-4474.
In this study the authors elucidated the transcriptional network responsible for coordinating changes in gene expression following H_2O_2 exposure in *E. histolytica*.

45. •• Husain A, Sato D, Jeelani G, Soga T, Nozaki T: **Dramatic increase in glycerol biosynthesis upon oxidative stress in the anaerobic protozoan parasite *Entamoeba histolytica***. *PLoS Negl Trop Dis* 2012, **6**:e1831.

In this study the authors highlighted several unexpected oxidative stress-induced metabolomic changes, and discussed their relevance in relation to oxidative stress mechanisms in *E. histolytica*.

46. Fahey RC, Newton GL, Arrick B, Overdank-Bogart T, Aley SB: ***Entamoeba histolytica*: a eukaryote without glutathione metabolism**. *Science* 1984, **224**:70-72.

47. Gillin FD, Diamond LS: ***Entamoeba histolytica* and *Giardia lamblia*: effects of cysteine and oxygen tension on trophozoite attachment to glass and survival in culture media**. *Exp Parasitol* 1981, **52**:9-17.

48. Jeelani G, Husain A, Sato D, Ali V, Suematsu M, Soga T, Nozaki T: **Two atypical L-cysteine-regulated NADPH-dependent oxidoreductases involved in redox maintenance, L-cystine and iron reduction, and metronidazole activation in the enteric protozoan *Entamoeba histolytica***. *J Biol Chem* 2010, **285**:26889-26899.

49. Husain A, Jeelani G, Sato D, Nozaki T: **Global analysis of gene expression in response to L-cysteine deprivation in the anaerobic protozoan parasite *Entamoeba histolytica***. *BMC Genomics* 2011, **12**:275.

50. Gillin FD, Diamond LS: ***Entamoeba histolytica* and *Giardia lamblia*: growth responses to reducing agents**. *Exp Parasitol* 1981, **51**:382-391.

51. Husain A, Sato D, Jeelani G, Mi-ichi F, Ali V, Suematsu M, Soga T, Nozaki T: **Metabolome analysis revealed increase in S-methylcysteine and phosphatidyl isopropanolamine synthesis upon L-cysteine deprivation in the anaerobic protozoan parasite *Entamoeba histolytica***. *J Biol Chem* 2010, **285**:39160-39170.

52. Rébeillé F, Jabrin S, Bligny R, Loizeau K, Gambonnet B, Van Wilder V, Douce R, Ravanel S: **Methionine catabolism in Arabidopsis cells is initiated by a gamma-cleavage process and leads to S-methylcysteine and isoleucine syntheses**. *Proc Natl Acad Sci USA* 2006, **103**:15687-15692.

53. Chiang PK, Gordon RK, Tal J, Zeng GC, Doctor BP, Pardhasaradhi K, McCann PP: **S-Adenosylmethionine and methylation**. *FASEB J* 1996, **10**:471-480.

54. de Vienne D, Bost B, Fievet J, Zivy M, Dillmann C: **Genetic variability of proteome expression and metabolic control**. *Plant Physiol Biochem* 2001, **39**:271-283.

55. •• Makiuchi T, Nozaki T: **Highly divergent mitochondrion-related organelles in anaerobic parasitic protozoa**. *Biochimie* 2013 http://dx.doi.org/10.1016/j.biochi.2013.11.018.
This review summarized and discussed the diversity in the contents and functions of the mitochondrion-related organelles (MROs) from anaerobic parasitic protozoa.